Journal of Clinical Laboratory Analysis 23 : 7–13 (2009)

First Production of Fluorescent Anti-Ribonucleoproteins Conjugate for Diagnostic of Rabies in Brazil

Graciane Maria Medeiros Caporale,[1] Andréa de Cássia Rodrigues da Silva,[1] Zélia Maria Pinheiro Peixoto,[1] Luciana Botelho Chaves,[1] Maria Luiza Carrieri,[1] and Ruth Camargo Vassão[2]*

[1]Rabies Diagnostic Laboratory, Instituto Pasteur de São Paulo, São Paulo, Brazil
[2]Genetics Laboratory, Instituto Butantan, São Paulo, Brazil

The laboratory tests recommended by the World Health Organization for detection of rabies virus and evaluation of specific antibodies are performed with fluorescent antibodies against the virus, the ribonucleoproteins (RNPs), or by monoclonal antibodies. In this study, we purified the rabies virus RNPs for the production of a conjugate presenting sensibility and specificity compatible with commercial reagents. The method employed for the purification of RNPs was ultracentrifugation in cesium chloride gradient, the obtained product being used for immunizing rabbits, from which the hyperimmune sera were collected. The serum used for conjugate production was the one presenting the highest titer (1/2,560) when tested by indirect immunofluorescence. The antibodies were purified by anion exchange chromatography (QAE-Sephadex A-50), conjugated to fluorescein isothiocyanate and separated by gel filtration (Sephadex G-50). The resulting conjugate presented titers of 1/400 and 1/500 when assayed by direct immunofluorescence (DIF) and simplified fluorescence inhibition microtest, respectively. Sensibility and specificity tests were performed by DIF in 100 central nervous system samples of different animal species, presenting 100% matches when compared with the commercial reagent used as standard, independent of the conservation state of the samples. The quality reached by our conjugate will enable the standardization of this reagent for use by the laboratories performing diagnosis of rabies in Brazil, contributing to the intensification of the epidemiological vigilance and research on this disease. J. Clin. Lab. Anal. 23 : 7–13, 2009.

Key words: rabies; diagnostic; immunofluorescence; conjugate; purification; ribonucleoproteins

INTRODUCTION

According to data from the World Health Organization, approximately 55,000 cases of human rabies occur every year in the world, most of them in developing countries (1). Laboratorial diagnostic tests must be fast and precise, because the results influence not only the medical decision of initiating prophylactic treatment, but also the elaboration of control measures of a potential epizooty in a community (2). The test, considered as a gold standard for laboratory diagnosis is direct immunofluorescence (DIF) (3), performed in central nervous system (CNS) of animals suspected of infection by the virus and in tests using cell cultures, either for viral isolation or for the evaluation of neutralizing antibodies' titers.

Since 1933, proteins have been labeled with chemical stains (4); however, only in 1958, Goldwasser and Kissling adapted the fluorescent antibodies technique to the identification of rabies virus antigens (5).

Dean and Abelseth (6), in 1973, used inactivated rabies infected mice brain suspensions as a source of antigens for immunization of animals, and, starting from the hyperimmune sera, obtained concentrated specific antibodies through treatment with ammonium

*Correspondence to: Ruth Camargo Vassão, Genetics Laboratory, Instituto Butantan, Av. Vital Brasil, 1500 CEP 05503-900 São Paulo, Brazil. E-mail: ruthvassao@butantan.gov.br

Received 6 September 2008; Accepted 25 September 2008
DOI 10.1002/jcla.20275
Published online in Wiley InterScience (www.interscience.wiley.com).

suphate (7). The conjugates used in laboratorial diagnosis of rabies, performed by DIF, are obtained from hyperimmune sera of animals immunized with vaccines, viral particles, or purified ribonucleoproteins (RNPs). In 1975, the introduction of hybridomas by Kohler and Milstein (8) enabled the use of monoclonal antibodies cocktails for the production of pure and specific conjugates (9).

The rabies virus is composed of two major structures: the nucleocapside, containing the RNPs formed by phosphoprotein (P), RNA-polymerase (L), and the nucleocapsid protein (N), which is the most genetically conserved and the viral envelope, which contains the more variable protein, the glycoprotein (10–12).

Purified immunoglobulins G (IgG) are rapidly obtained by anion exchange chromatography with QAE-Sephadex A-50 (Sweden) (13) and used for the production of fluorescent conjugates, through labeling with fluorescein isothiocyanate (FITC), the most used reagent for DIF owing to its stability and characteristic fluorescence (6).

The national standardization of reagents and procedures for rabies diagnosis is recommended for the establishment of a modern epidemiological vigilance system for infectious diseases, based fundamentally on the achievement of the laboratorial diagnosis methods.

The aim of this study was the production of fluorescent anti-RNP conjugate, with high sensitivity and specificity, to be used in the laboratory diagnosis of rabies by DIF.

MATERIALS AND METHODS

Rabies Virus: Obtention of RNP

For the viral particles' propagation, 24 cell culture flasks of 225 cm^2 each were used. In each flask, 9 mL of 10^6/mL baby hamster kidney (BHK-21) cells and 9 mL of Pasteur virus (PV) suspension with a $10^{4.6}$ focus forming dosis (FFD) titer were added, and the volume was completed with 72 mL of Eagle's minimum essential medium supplemented with 10% fetal bovine serum. The cultures were incubated for 48 hr at 34°C and the supernatants were discarded. The adhered cells were washed with 40 mL sterile phosphate buffered saline (PBS), collected with a cell scraper suspended with ice-cold 0.5 M sodium chloride/tris-HCl (NT) buffer pH 7.6 and centrifuged twice at 900*G*, 4°C for 10 min.

Cell lysis was done by adding 5 mL of ice-cold, sterile, deionized water containing 1,000 units of aprotinin (14) for each 2 mL of cell lysate. This mixture was incubated for 1 hr at 4°C and centrifuged at 1,000*G*, 4°C for 20 min. The supernatant was collected and this procedure was repeated twice. The final supernatant was then centrifuged at 12,000*G*, 4°C for 10 min.

RNP Purification

The RNP was purified by ultracentrifugation in cesium chloride (CsCl) gradient, as described by Dietzschold (14).

For each 3–4 mL of RNP containing supernatant, 2 g of CsCl were added in 5 mL polycarbonate tubes. The final volume was adjusted to 5 mL with pH 7.6 NT buffer and centrifuged at 150,000*G* for 18 hr at 4°C. The bands (approximately 0.7 mL) were collected, pooled, and dialyzed against pH 7.6 NT buffer for 24 hr at 4°C (15).

RNP Presence Confirmation

A sample of purified RNP was loaded in a 12.5% SDS-PAGE gel, with a 5% stacking for the detection of viral proteins by electrophoresis (16). The RNP was also analyzed by electron microscopy, with 2% pH 5.0 ammonium molybdate negative contrast.

RNP Concentration Measurements

The protein concentration was measured by spectrophotometry, using the 260/280 nm absorbance ratio (17), and expressed as mg/mL.

Immunization Schedule

Four New Zealand male rabbits of 3-months old, weighting around 3 kg were immunized for the obtention of anti-RNP hyperimmune sera. The immunization schedule consisted of four doses with 7 days interval, followed by two boosters with 14 days interval (days 0, 7, 14, 21, 35, and 49). The antigen was prepared by mixing 200 μg RNP in 1 mL PBS with 0.5 mL complete Freund adjuvant for the first dose, and incomplete for the following injections, all given subcutaneously at several sites. Ten days after the last booster, blood samples were collected from the auricular vein and used for titration by indirect immunofluorescence (IIF) (18).

IgG Purification

For the purification and production of the conjugate, the sample presenting the highest titer was selected. A 25 mL serum sample was diluted in equal volume of ethylenediamine buffer pH 7.0 (TED) and dialyzed against the same buffer for 24 hr (7). The IgG were then purified by anion exchange chromatography on a Sephadex QAE-50 column previously equilibrated with TED, and eluted with a saline gradient (13). The collected fractions were read at 280 nm on a spectrophotometer and only those presenting an optical density above 1.0 were pooled and used for the precipitation step. An equal volume of saturated ammonium sulphate

was added to the sample, which was then centrifuged at 1,500*G*, 4°C for 10 min. The resulting pellet was diluted in 1 mL PBS and dialyzed for 24 hr against the same buffer. The protein concentration was estimated by spectrophotometry at 280 nm (17).

IgG-Isothiocyanate Conjugation

To each volume of IgG solution, 10% of 0.5 M pH 9.0 sodium carbonate/bicarbonate buffer and 1 mg of FITC/100 mg of protein were added. The resulting solution was kept at 4°C for 24 hr under constant stirring and the labeled antibodies were then separated by gel filtration on a Sephadex G-50 column equilibrated and eluted with PBS pH 7.0.

Titration of the Conjugate

The titer of the anti-RNP conjugate was determined for its application in DIF (19), simplified fluorescence inhibition microtest (SFIMT) (20) and rapid fluorescent focus inhibition (21). For the DIF, the conjugate titration was done in slides imprinted with CNS of rabies-infected mice. Two fold dilutions were done from 1:10 to 1:640. The slides were incubated for 30 min at 37°C in a humid chamber, washed with PBS and distilled water, and, after drying, observed under the fluorescence microscope (Leica, Germany) with 400 × magnification.

For the serum neutralization in cell cultures, 96 flat bottom wells micro plates were used. In each well, 50 μL of BHK-21cells (10^6 cells/mL) and were added to 50 μL of PV strain, in a concentration that promotes 80–100% cell infection ($10^{4.6}$ FFD50). Noninfected BHK-21 cells were used as negative controls. After 24 hr incubation at 37°C and 5% CO_2, the wells supernatants were aspired and the plates were placed on ice and fixed with 80% cold acetone for 15 min.

The conjugate was diluted from 1:5 to 1:2,560 in PBS containing Evans Blue (0.1%) and 40 μL of each dilution was transferred to eight wells containing either infected or not cells. The plate was incubated for 1 hr at 37°C, washed in PBS, dried, and observed under the fluorescence microscope with 100 × magnification.

Relative Sensitivity and Specificity Assays

One hundred CNS samples were selected for this assay from those submitted to rabies diagnosis by Pasteur Institute Laboratory. These samples were in different states of conservation and were kept at −80°C. In addition, they were of different origin: 39 bovine, 20 equine, 17 canine, 1 simian, 4 feline, 3 caprine, 1 swine, 5 ovine, 2 asinine, and 8 human. For these assays the samples were analyzed without knowledge of previous results.

For the DIF (19), three slides of each sample were prepared with brain tissue imprints, using always the same fragment. The slides were fixed with ice-cold acetone for 45 min in the freezer at −20°C. The anti-RNP conjugate, diluted at the previously determined concentration was added to two slides, whereas the commercial anti-nucleocapside conjugate (Bio-Rad, Foster City, CA; lot 3A0041-2004) was used as a standard on the third slide.

Slides with PV-infected BHK-21 and N2A cells as well as human, canine, and bovine CNS imprints with their respective negative controls were selected to be analyzed by confocal microscopy.

RESULTS

RNP Purification

The volume obtained after lysis of the infected cells was of 28 mL and after purification of 4 mL. The purified RNP concentration was of 5.8 mg/mL (Table 1).

In Figure 1 we can observe a clear band with an approximate molecular weight of 57 kDa, whereas Figure 2 shows an electron micrography of filaments, characteristic of RNP.

Anti-RNP Serum, Purification, and Quantification of IgG

The hyperimmune serum selected for IgG purification showed intense fluorescence with a dilution of 1:2,560 when assayed by IIF.

The volume obtained after precipitation of the IgG was of 60 mL, with a concentration of 32.7 mg/mL.

Anti-RNP Conjugate Titration

The titer obtained for DIF was of 1:400 and of 1:500 for serum neutralization in BHK-21 cell culture.

Relative Sensitivity and Specificity Assays

The results obtained by DIF in CNS samples of different species were 100% concordant for the two conjugates (Table 2) and also matched previous tests.

TABLE 1. Volumes before and after the purification and concentration of proteins

Step	RNPs
Volume before purification	28.0 mL
Volume after purification	4.0 mL
Protein concentration after purification	5.8 mg/mL

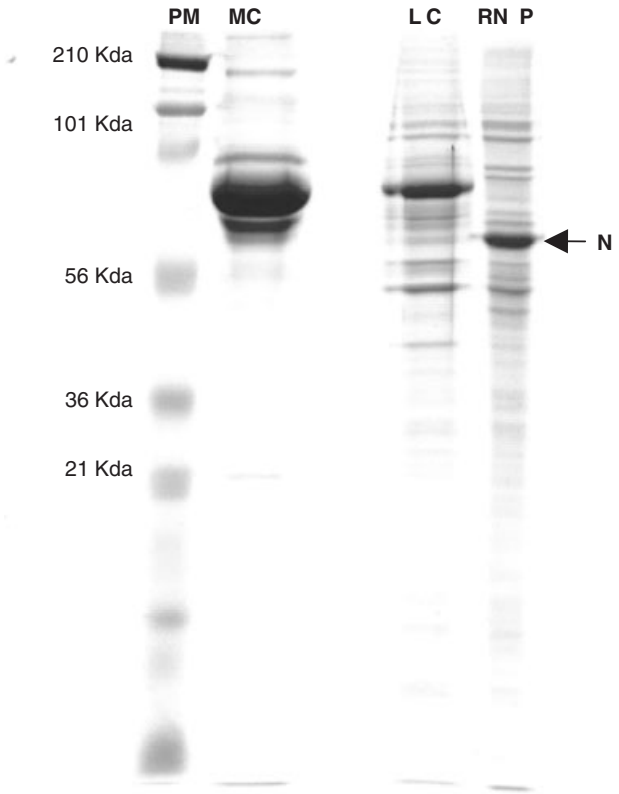

Fig. 1. Polyacrylamide gel (SDS-PAGE). MW, molecular weight markers; CM, culture medium; CL, cell lysate; RNPs, ribonucleoproteins; and N, nucleoproteins, after purification on cesium chloride (CsCl).

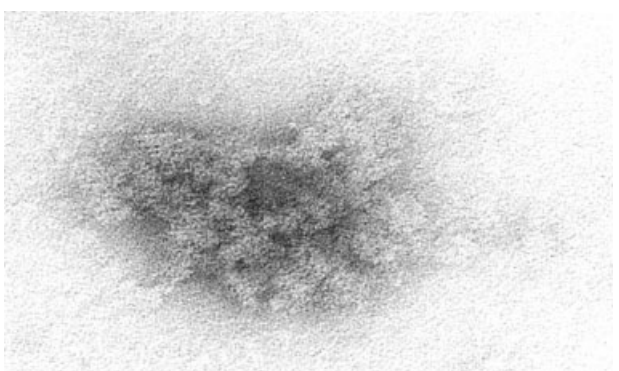

Fig. 2. Photomicrography of the ribonucleocapside fraction after purification.

Figures 3–8 show BHK-21 and N2A cells as well as brain imprints infected with different virus samples and the respective negative controls, developed by DIF with the conjugate, enabling the observation of well-defined Negri bodies, intense fluorescence and lack of nonspecific fluorescence in the negative controls.

TABLE 2. DI results of central nervous system samples according to the animal species and the anti-rabies vírus conjugates employed

	Anti-RNPs[a] conjugate		Commercial[b] conjugate		
Species	Negative	Positive	Negative	Positive	Total
Bovine	9	30[c]	9	30[c]	39
Canine	9[d]	8	9[d]	8	17
Caprine	3	0	3	0	3
Human	0	8[e]	0	8[e]	8
Feline	3	1	3	1	4
Equine	3	17[f]	3	17[f]	20
Simian	1	0	1	0	1
Muar	0	2	0	2	2
Ovine	0	5	0	5	5
Suine	1	0	1	0	1
Total	29	71	29	71	100

[a]Anti-RNPs conjugate, batch: Instituto Pasteur—2004 (L.IP.CA-RNPs/04).
[b]Anti-nucleocapside conjugate batch: 3A0041-2004 (BIO-RAD), used as standard.
[c]Three samples—samples that presented high degree of putrefaction.
[d]Two samples—samples that presented high degree of putrefaction.
[e]Three samples—samples that presented high degree of putrefaction.
[f]Eight samples—samples presenting few Negri bodies.

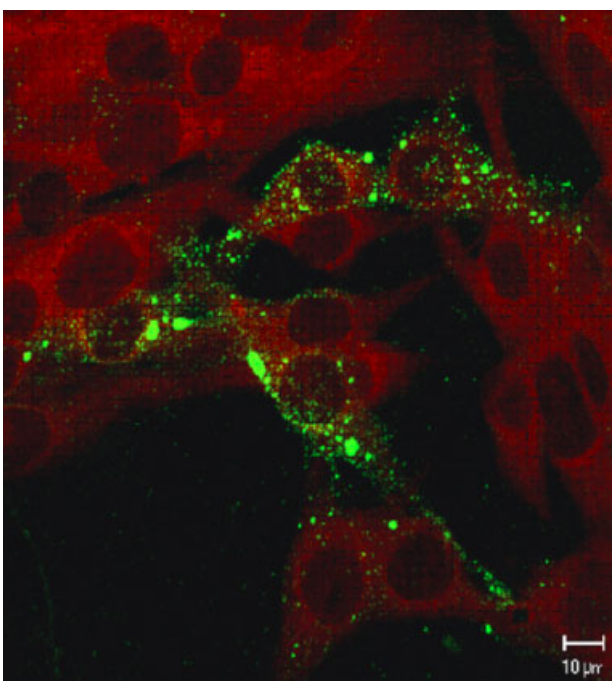

Fig. 3. BHK-21 cells infected with fixed PV-conjugate L.IP. CA-RNPs/04.

DISCUSSION

The DIF is fast and precise but depends on the sensitivity and specificity, partly on affinity, titer, and proper linking of the fluorochrome to the

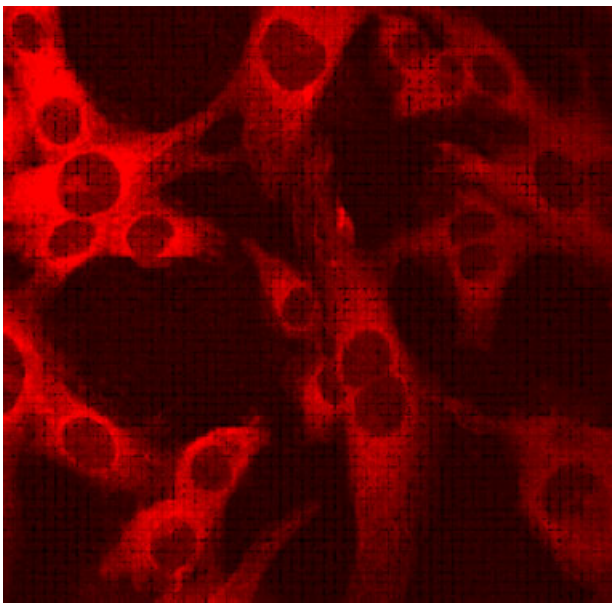

Fig. 4. BHK-21 cells-negative control conjugate L.IP. CA-RNPs/04.

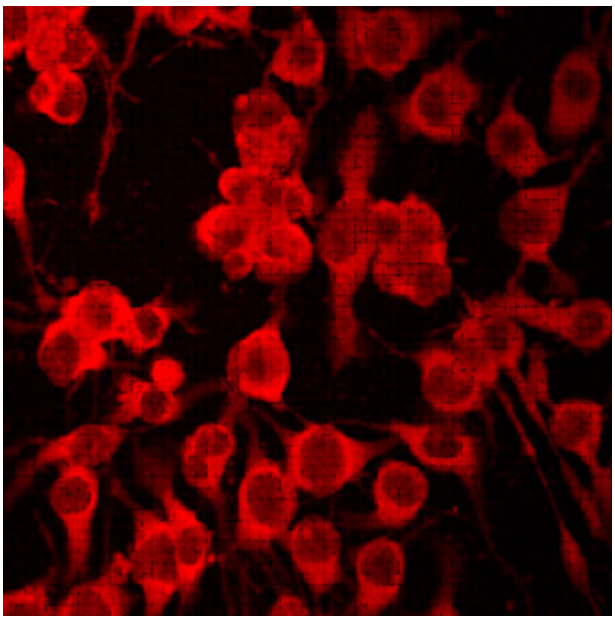

Fig. 6. N2A cells-negative control conjugate L.IP. CA-RNPs/04.

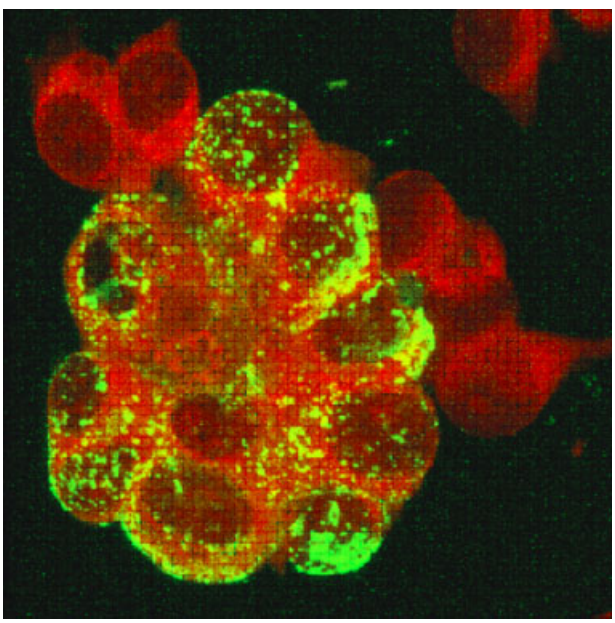

Fig. 5. N2A cells infected with fixed CVS-conjugate L.IP. CA-RNPs/04.

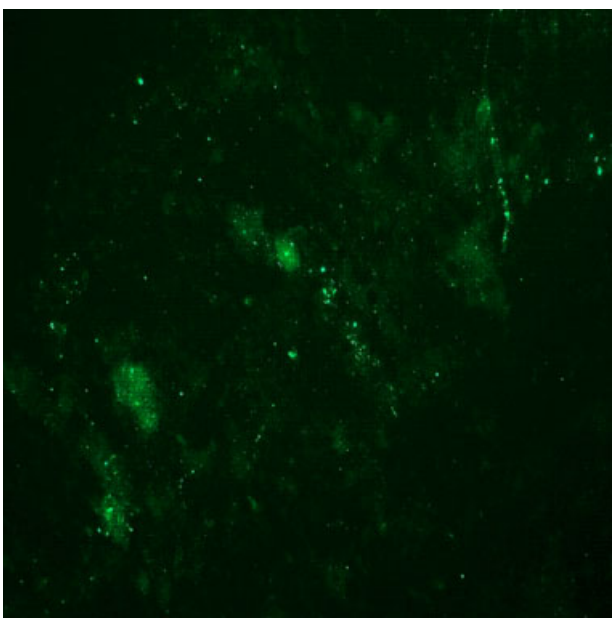

Fig. 7. Positive CNS imprint—canine conjugate L.IP. CA-RNPs/04.

antibodies (9). The quality of fluorescence depends on the method of immunogen production, and the presence of nonrabies antigens can be avoided using viral preparations from homologous tissues or purified antigens (22).

In this study, the use of purified RNP as a suspension to immunize animals for the obtention of hyperimmune sera for the production of fluorescent conjugates was determinant to reach the results demonstrated by a 100% match with the commercial anti-nucleocapsid conjugate in the sensitivity and specificity tests. Such results could not be obtained in the production of the first batch of fluorescent conjugate from Peru, using antibodies raised against a vaccine produced in VERO cells, where nonspecific fluorescence was observed in DIF (23).

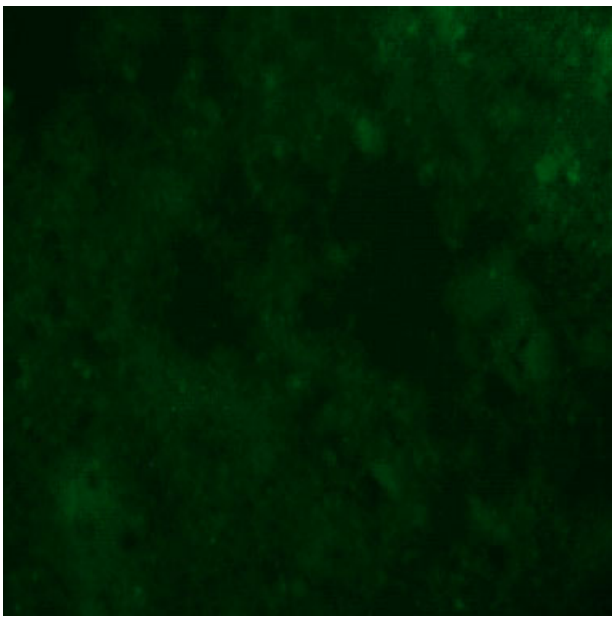

Fig. 8. Negative CNS imprint—canine conjugate L.IP. RNPs/04.

The electron micrography data from the purified extract confirmed the presence of RNP with the characteristic spiraled shape, similar to those previously observed using rabies virus nucleocapsid produced in cell cultures (24).

Although some minor bands corresponding to cell lysate proteins were observed on the SDS-PAGE, suggesting only partial purification of the RNP, no interference on the specificity was detected. This fact is probably owing to the high concentration of proteins (5.8 mg/mL) in the purified extract, enabling high dilution factors to prepare the 200 μg immunizing doses. Thus, the possible presence of nonspecific antibodies raised against nonviral proteins present in the extract did not interfered in the conjugate specificity.

The IgG concentration obtained after the anion exchange chromatography of the hyperimmune sera was satisfactory, because the amount of antibodies was superior to the one recommended for FITC conjugation, which is of 20 mg/mL (7). These yields are in agreement with the results obtained by Atanasiu et al. (25) in 1974 when the methodology above was compared with ammonium sulphate precipitation and all the data indicated higher IgG concentration in the samples purified by anion exchange chromatography.

The 1:400 and 1:500 titers obtained for DIF and SFIMT, respectively, were fairly high, considering that the reagent can be diluted 20 folds more than the commercial one. However, these results do not eliminate the need for titration of each batch of conjugate in each laboratory, independently from the titer obtained from the producing laboratory, because the titers can vary depending on the characteristics of the microscope and the intensity of the light source, as demonstrated in a inter laboratorial study, where titers ranged between 1:4 and 1:64 depending on the accessories used (26).

Beside titers, other criteria that must be analyzed for the evaluation of conjugates quality are specific fluorescence intensity, amount of inclusions (Negri bodies), and the absence of fluorescence in negative controls (27).

The 100% match when comparing our antibody with the commercial one when using DIF in the sensitivity and specificity assays showed that the technique did not suffer any kind of interference, even using CNS samples from different animal species and, although other studies demonstrated that DIF efficacy could be compromised by the loss of sensitivity when the samples analyzed where decomposed (28,29), this did not occur with our conjugate, even in the samples presenting scarce amounts of Negri bodies or high levels of decomposition.

These results indicate that conjugates produced from sera obtained from animals immunized with purified RNP can be considered as highly specific because they bind to these proteins in intra-cytoplasmatic inclusions in infected cells, enabling the laboratorial diagnosis of rabies in samples with different states of conservation (30).

According to information from the General Coordination of Laboratories (CGLab) from the Brazilian Ministry of Health, 32,000 rabies diagnostic tests and 18,000 serological tests for titration of neutralizing antibodies are performed every year, both using DIF, visualized by fluorescent conjugates.

Considering the volume and titers obtained, it would be possible to supply the annual need for rabies laboratory tests of the country, with an estimation of a capacity to perform 71,820 diagnostic assays and 18,750 serological tests, whereas with the same volume of commercial conjugate, the estimative would be of 3,580 and 750 tests, respectively.

The purification of RNPss for the obtention of hyperimmune serum, as well as the efficacy reached in all the steps of the conjugate production resulted in a high quality product that matched all the criteria considered important for such a reagent. The production of a conjugate following the methodology above might contribute to the national standardization for the use of this reagent for the laboratory diagnosis of rabies, increasing the capacity for epidemiologic vigilance and scientific research in Brazil.

ACKNOWLEDGMENTS

Ivanete Kotait, Karin Corrêa Scheffer Ferreira, Patrick Jack Spencer.

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